



Refined genome-wide comparative map of the domestic horse, donkey and human based on cross-species chromosome painting: insight into the occasional fertility of mules

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Abstract

We have made a complete set of painting probes for the domestic horse by degenerate oligonucleotide-primed PCR amplification of flow-sorted horse chromosomes. The horse probes, together with a full set of those available for human, were hybridized onto metaphase chromosomes of human, horse and mule. Based on the hybridization results, we have generated genome-wide comparative chromosome maps involving the domestic horse, donkey and human. These maps define the overall distribution and boundaries of evolutionarily conserved chromosomal segments in the three genomes. Our results shed further light on the karyotypic relationships among these species and, in particular, the chromosomal rearrangements that underlie hybrid sterility and the occasional fertility of mules.

Introduction

The domestic horse (*Equus caballus*, $2n = 64$) and the donkey (*Equus asinus*, $2n = 62$) are members of family Equidae that last shared a common ancestor 1.9–2.3 million years ago (Okenfull *et al.* 2000). Cytogenetic studies of horse and donkey have been prompted by an interest in the biological basis of the sterility of their hybrid offspring. Although diploid numbers of the horse and donkey differ only by one pair of chromosomes, comparative cytogenetic studies have reported considerable differences in arm number (NF) and G-, R- and C-banding patterns, indicating that extensive chromosomal rearrangements distinguish the karyotypes of

horse and donkey (Ryder *et al.* 1978). There have been several efforts to standardize the horse karyotype (Ford *et al.* 1980, Richer *et al.* 1990, Bowling *et al.* 1997). Although most chromosomes of the horse and donkey can be identified by banding patterns, conventional cytogenetic approaches have proved incapable of resolving the precise correspondence between the horse and donkey karyotypes. This is due to the complexity of chromosomal rearrangements that have occurred during their speciation (Ryder *et al.* 1978, Raudsepp & Chowdhary 1999).

Chromosome painting (Pinkel *et al.* 1988) is a special application of the fluorescence *in-situ* hybridization technique in which a complex mixture of DNA sequences, representative of a

single chromosome or chromosomal subregion, is used as a probe to visualize homologous elements in metaphase spreads and interphase nuclei. During recent years, cross-species chromosome painting (Wienberg *et al.* 1990, Scherthan *et al.* 1994, Yang *et al.* 1995) has become the method of choice for elucidating homologous chromosomal regions between both closely-related and distantly-related species and, in particular, between species with highly rearranged karyotypes. In an attempt to resolve the karyotypic relationships between the horse and donkey, Raudsepp & Chowdhary (1999) used chromosome-specific painting probes from microdissected metacentric and submetacentric autosomes (ECA1–13) and the sex chromosomes of the horse to delineate homologous chromosomal segments in the donkey. However, these paints only represented approximately half of the equine genome and thus the comparative chromosome map remains incomplete.

The success of the human genome project has prompted international efforts to map the horse genome using various approaches. The first attempt involved comparative chromosome painting of horse chromosomes using whole chromosome-specific probes of human (Raudsepp *et al.* 1996). This map consists of 43 homologous segments and covers most of the horse chromosomes, with three chromosomes (ECA chromosomes 12, 27 and 31) and two chromosomal arms (6p and 13p) of the horse left uncharted. Importantly, the majority of the conserved segments await confirmation to the human karyotype by 'reverse' painting of horse painting probes onto human chromosomes. This was followed by the generation of comparative gene maps of the human and horse (Caetano *et al.* 1999, Milenkovic *et al.* 2002) that similarly do not adequately allow for the precise demarcation of horse/human homologous segments on human chromosomes. Most recently, the first generation radiation hybrid (RH) map of 730 equine markers has been developed (Chowdhary *et al.* 2003), greatly improving the resolution of the human–horse map. However, in contrast to these developments, the determination of direct homology between the donkey and human has been largely neglected, apart from the homologies involving HSA4 and HSA16q (Raudsepp *et al.* 1999).

In order to further clarify karyotypic relationships between the horse and donkey and also refine the comparative chromosome map between horse, donkey and human, we have made a complete set of chromosome-specific painting probes for the horse by degenerate oligonucleotide primed PCR (DOP-PCR, Telenius *et al.* 1992) amplification of flow-sorted chromosomes. These probes have been hybridized *in-situ* to metaphase preparations of these three species. The data enable the establishment of the number and types of all interchromosomal and some intra-chromosomal rearrangements that differentiate the karyotypes of these equids and provide insights into the chromosomal basis of the sterility of horse and donkey hybrids.

Materials and methods

Metaphase preparations

Horse metaphases were prepared from the E. Derm cell line purchased from ECACC (No. 88032803) and from a fibroblast culture KCB 94015 kindly provided by the Kunming Cell Bank of the Chinese Academy of Sciences, Yunnan 650223, PR China. Human metaphase preparations were made from PHA-stimulated peripheral blood cultures following standard procedures. Donkey metaphases were made from a male primary fibroblast culture derived from a skin biopsy (KCB 89010). A fibroblast culture of a female mule (*E. asinus* × *E. caballus*) was also used in this study. Previous studies had shown this hybrid to have a $2n = 63$ karyotype (Ryder *et al.* 1985). Horse chromosomes were identified according to the international standard nomenclature of the horse (Bowling *et al.* 1997) and donkey chromosomes were numbered following the most recent nomenclature for the donkey proposed by Raudsepp *et al.* (2000).

Flow sorting and generation of chromosome-specific paintprobes

Horse chromosomes were sorted on a dual laser cell sorter (FACStar Plus, Becton Dickinson)

as described (Yang *et al.* 1995). Chromosome-specific painting probes were made by degenerate oligonucleotide PCR (DOP-PCR) amplification of flow-sorted chromosomes following previously described methods (Telenius *et al.* 1992, Yang *et al.* 1995). DOP-PCR amplified chromosome-specific DNAs were labelled during secondary PCR by incorporating either biotin-16-dUTP, or fluorescein-12-dUTP or Cy3-dUTP.

Fluorescence in-situ hybridization (FISH)

Comparative chromosome painting was performed as previously described (Yang *et al.* 1997, 1999). In brief, 150 ng of biotin-labelled chromosome-specific paints were made up to 12 µl with hybridization buffer (50% deionized formamide, 10% dextran sulphate, 2 × SSC, 0.5 mol/L phosphate buffer, pH 7.3, and 1 × Denhardt's solution). The probes were denatured at 65°C for 10 min and then pre-annealed by incubation at 37°C for 15–60 min. Metaphase slides were denatured by incubation in 70% formamide/30% 2 × SSC (v/v) solution at 65°C for 1.5–2 min, quenched in ice-cold 70% ethanol, and dehydrated through a 70, 90 and 100% ethanol series. The pre-annealed paints were applied to slides, covered with 22 × 22-mm coverslips, sealed with rubber cement and incubated for 72 h at 37°C. Posthybridization washes involved two 5-min incubations in 50% formamide/50% 2 × SSC at 40°C followed by two 5-min incubations in 2 × SSC at 40°C. Biotin-labelled probes were visualized using Cy3-avidin (final concentration 2 ng/µl, Amersham). After detection, slides were mounted in Vectashield mounting medium with DAPI (4',6-diamidino-2-phenylindole, Vector Laboratories). Images were captured using the CytoVision system (Applied Imaging) and a Cohu CCD camera mounted on an Olympus BX-60 microscope as previously described (Yang *et al.* 1999). Hybridization signals were assigned to specific chromosome regions defined by DAPI- and/or G- banding.

For comparative painting among equid species (horse and donkey), the hybridization time was reduced to 16–24 h and the temperature for posthybridization washing was increased to 45°C. In cases where unambiguous identification of

chromosomes by DAPI banding was problematic, sequential G-banding (Seabright 1972) and 2–7-colour FISH were employed. Briefly, metaphase slides were baked at 65°C for 3 h and then treated with 0.01% trypsin containing 0.1 mmol/L EDTA for 8–12 min before staining with 2% Giemsa for 10 min. After image capture of G-banded metaphases, immersion oil and Giemsa stain were removed by passing the slides through 100% ethanol and 100% methanol (5 min in each instance). The slides were subsequently baked at 65°C for at least 1 h. The G-banded slides were denatured in a 70% formamide/30% 2 × SSC (v/v) solution at 60°C for 20–30 s. The hybridization, posthybridization washes, and detection conditions follow the procedure outlined above. For multicolour FISH, probes were combinatorially labelled with biotin-, FITC- and Cy3-dUTP following the labelling scheme proposed by Ried *et al.* (1992) and visualized with avidin-Cy5, rabbit-anti-FITC and FITC-conjugated goat-anti-rabbit antibodies.

Results

Flow karyotype of the domestic horse (KCB94015)

In order to generate a complete set of chromosome-specific paints for the horse, metaphase chromosome suspensions were prepared from two horse cell lines (KCB 94015 and E. Derm), stained with Hoechst 33258 and chromomycin A3, and then subjected to flow sorting. Figure 1 shows the flow karyotype of the cell line KCB94015. The E. Derm line has a similar, but less-well resolved, flow karyotype (not shown). Although not all chromosomes of the horse are resolved to separate peaks, we were able to develop a complete set of chromosome-specific paints for the horse by controlling the size of sorting gates, in conjunction with single-chromosome sorting and multicolour FISH (Figure 2a). The horse flow karyotype was characterized by assigning the paint from each flow peak onto DAPI- or G-banded horse chromosomes. This was followed by further verification after painting on human chromosomes using the previously established human–horse homology as a guide (Raudsepp *et al.* 1996, Chowdhary *et al.* 2003).

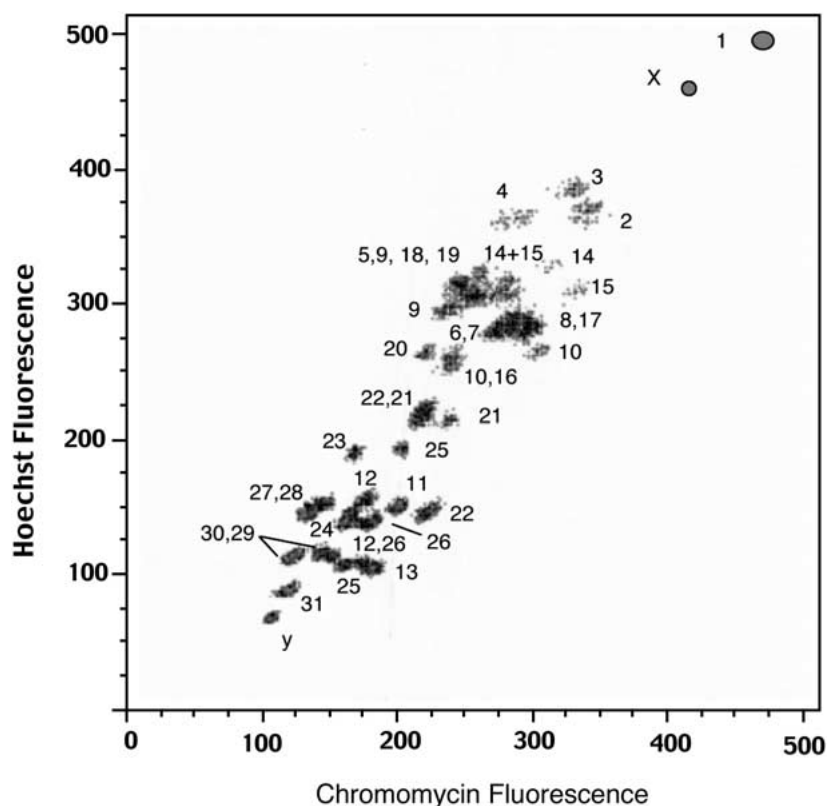


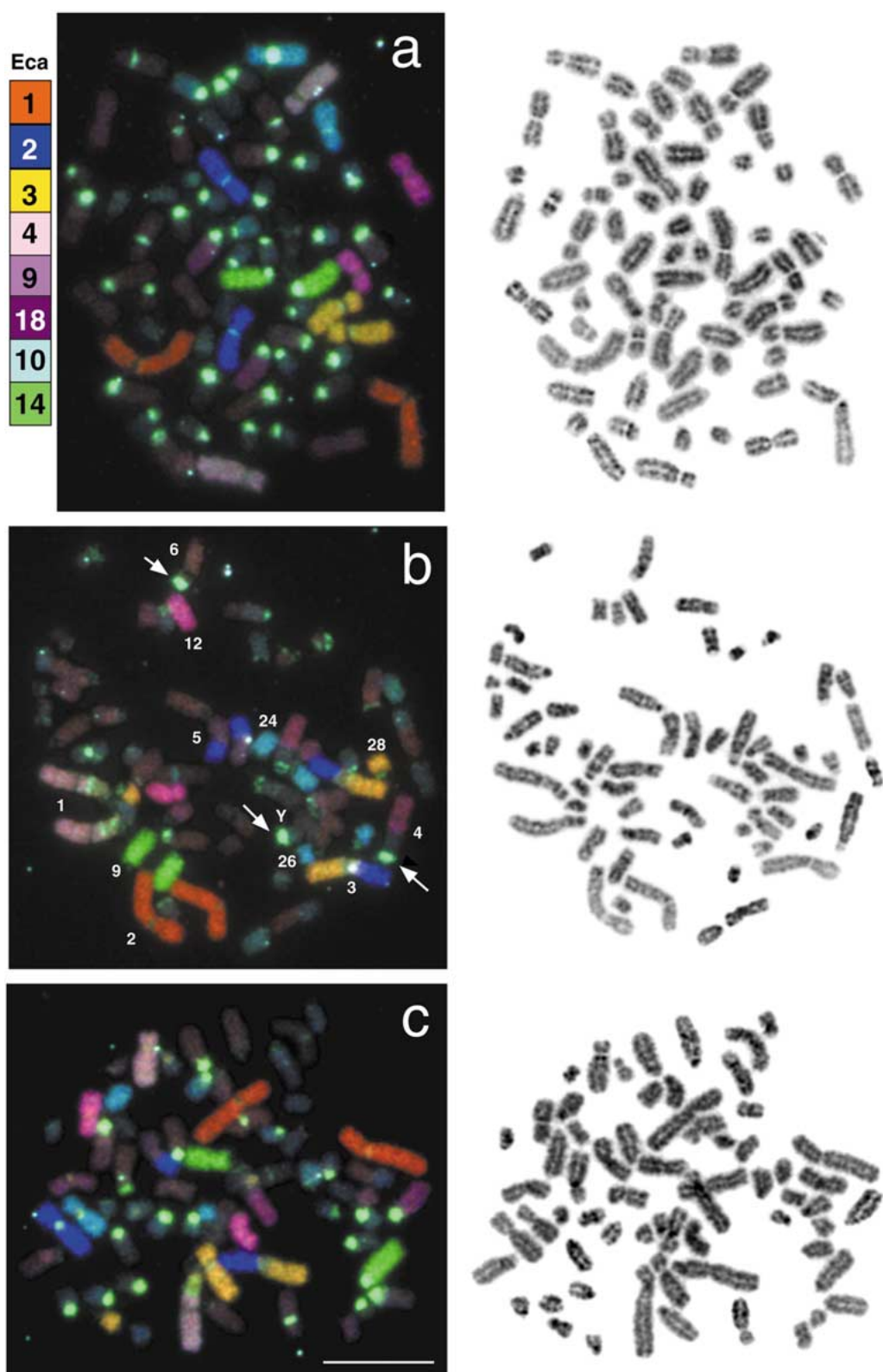
Figure 1. Flow karyotype of a domestic horse cell line. The relative positions of the X and chromosome 1 are marked in this figure since they were included in a separated data file when sorted.

Establishment of chromosomal correspondence between horse and donkey

The entire set of horse paints were hybridized onto donkey and mule metaphases. Eighteen horse chromosome paints (ECA 1, 7, 9, 11–17, 22, 23, 26, 27, 29, 30, X and Y) each delineate one entire donkey chromosome; seven probes (ECA 18–21, 24, 25, 28) painted one homologous segment (i.e. part of a donkey chromosome), while six probes (ECA 2, 3, 5, 6, 8 and 10) each gave a signal on two different donkey

chromosomes. The ECA 4 and 31 probes each gave two signals on donkey chromosome 1. Probes from horse acrocentric chromosomes produced strong cross-hybridization signals to the centromeric regions of horse acrocentrics, but only slight cross-hybridization to the donkey acrocentrics (Figure 2). In total, the 33 horse painting probes identified 41 conserved segments in the donkey genome. The genome-wide chromosomal correspondence is summarized on a G-banded karyotype of a female mule (Figure 3).

Figure 2. Simultaneous hybridization of combinatorially-labelled horse chromosomes 1, 2, 3, 4, 9, 10, 14 and 18 probes onto metaphases of the horse (a), donkey (b) and mule (c). The DAPI-banded metaphases are shown to the right of each FISH image. The colour of each horse probe is indicated to the left of horse metaphase (a). The identities of homologous donkey chromosomes are indicated in (b) with ECA 1 = EAS 2, ECA 2 = EAS 3p + EAS 5q, ECA 3 = EAS 3q + EAS 28, ECA 4 = EAS 1q, ECA 9 = EAS 12, ECA 10 = EAS 24 + EAS 26, ECA 14 = EAS 9, ECA 18 = EAS 4q. Note that horse probes show strong cross-hybridization to the centromeric regions of the acrocentric chromosomes of the horse (a) and (c), but very slight cross-hybridization to their donkey homologues with the exception of occasionally strong cross-hybridization signals on EAS 4pter, EAS 6pter and Y (b, arrows). Scale bar = 10 µl.



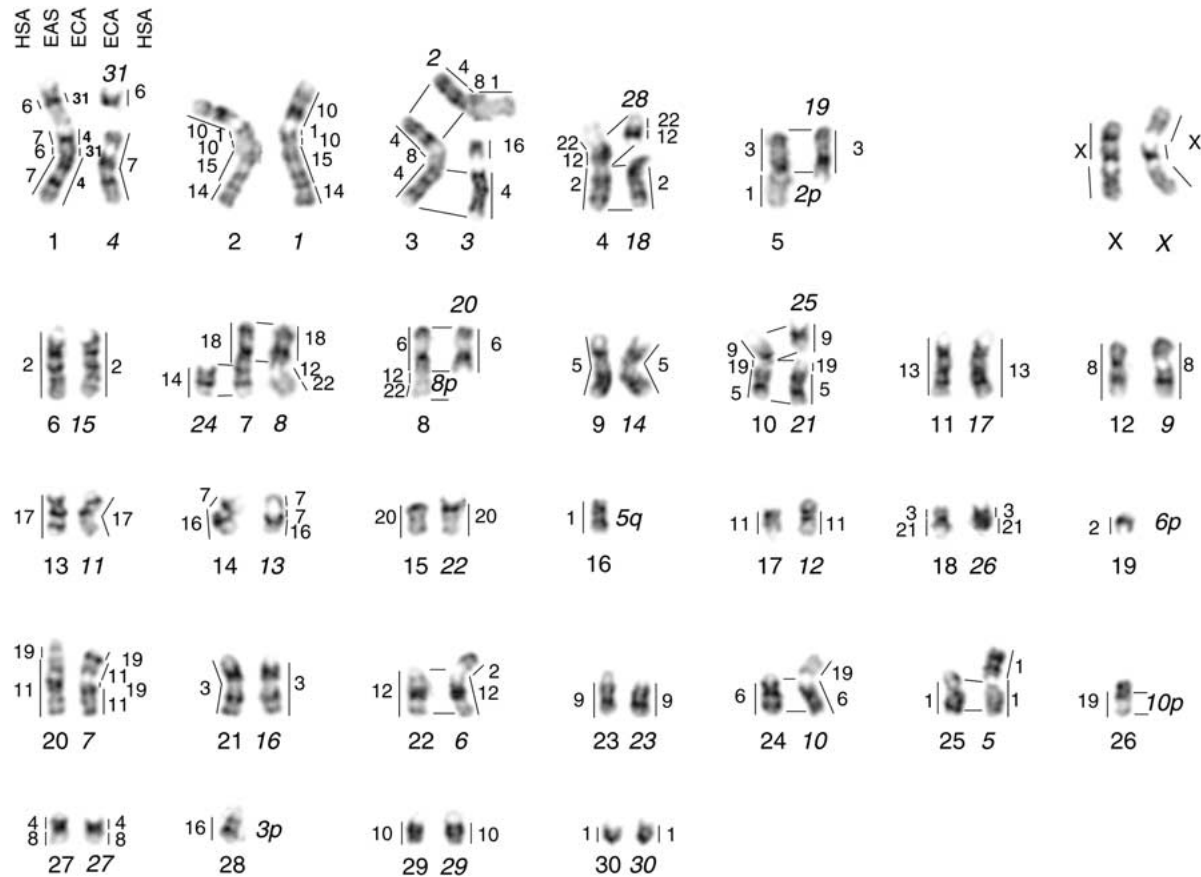


Figure 3. Genome-wide homology map of human, horse and donkey with the G-banded chromosomes of a female mule as reference. The regions of homology between horse and donkey were determined by sequential G-banding and multicolour FISH of the single metaphase spread used in the construction of this figure. The donkey chromosomes are shown to the left of each panel, while the corresponding horse chromosomes are shown to the right. The only exception to this arrangement concerns the panel comprising of donkey chromosome 7 which is shown in the middle with the horse homologues on either sides of it. The donkey chromosome numbers are given below the respective donkey chromosomes; horse chromosomes numbers are given in italics. The hybridization results of human probes are indicated to the left of each donkey chromosome and to the right of each horse chromosome. Note that ECA4 and 31 are each homologous to two segments on EAS1.

Painting human chromosomes with horse probes

Paints specific for the 31 horse autosomes plus the X chromosome identified 60 conserved segments (Figure 4). Human chromosomes 13, 15, 17, 18, 20, 21 and the X were each painted by probes from a single horse chromosome, with HSA 13, 17 and 20 showing a one-to-one correspondence to horse chromosomes 17, 11 and 22, respectively. These results are largely concordant with the previously published human–horse comparative maps based on the hybridization patterns of human onto horse and

comparative gene and RH mapping (Raudsepp *et al.* 1996, Milenkovic *et al.* 2002, Chowdhary *et al.* 2003). We did, however, identify several discrepancies that were further examined by repeated hybridization of human paints onto horse metaphases (Figure 5; see details in discussion).

Painting mule chromosomes with human probes

Although human paints have been used to delineate conserved chromosomal segments in the horse, they have not yet been applied to donkey chromosomes. The mule, as a hybrid offspring,

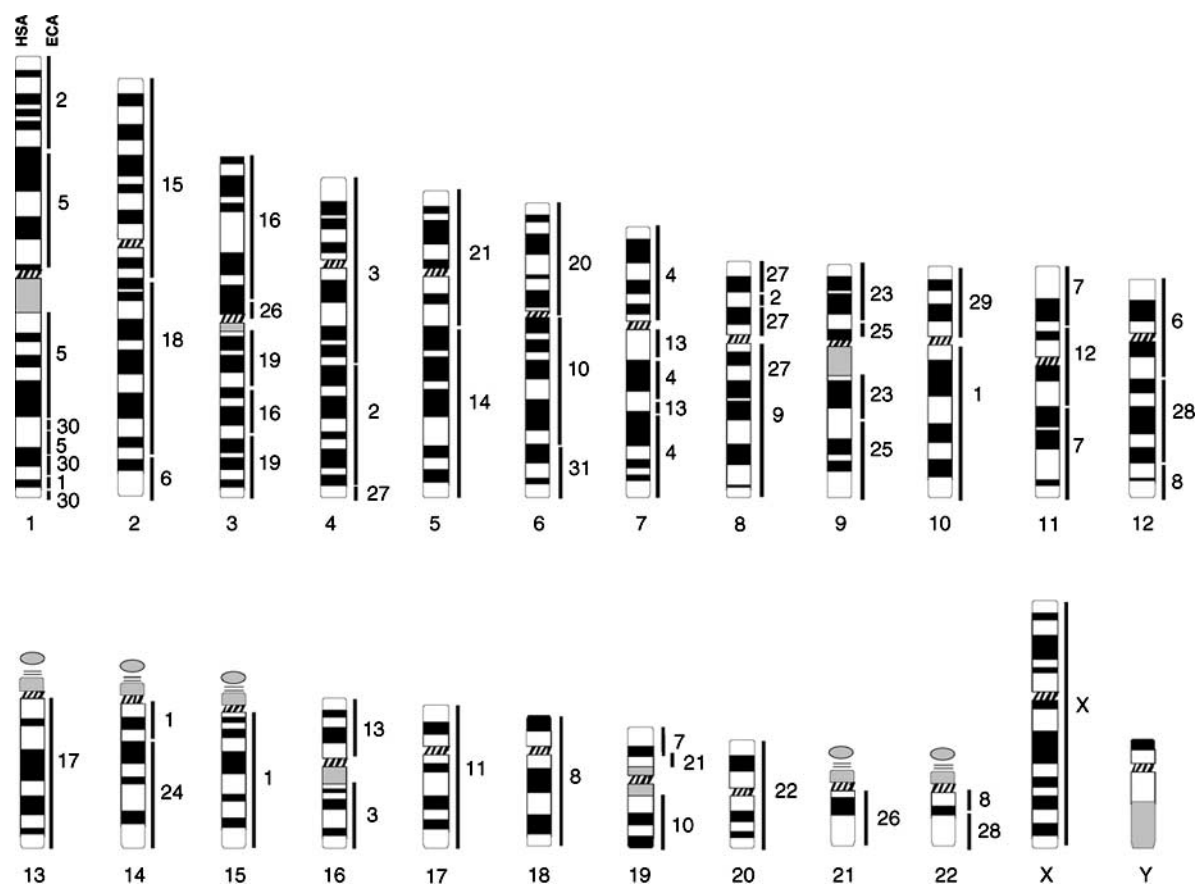


Figure 4. Summary of hybridization patterns of the full set of horse painting probes onto the human idiogram.

inherits one haploid set of chromosomes from each parent (i.e. the horse and donkey) and thus provides the ideal platform for simultaneous comparison of horse, donkey and human chromosomes within one hybridization. Painting probes specific for the 22 human autosomes and the X chromosome were hybridized onto mule metaphases. The 22 human autosomal probes delineate 51 conserved chromosomal segments in both the horse and donkey (Figures 3 & 5d). The HSA6/7 probe combination revealed an inversion between EAS1 and its horse homologues ECA 31 and 4, as did the HSA11/19 probe on EAS20 and its horse homologue ECA 7 (see details in Figure 3). In addition to the ancient syntenic associations of HSA7/16, 12/22, 14/15, the HSA 3/21 and 4/8, associations were also found in both the donkey and horse genomes. The hybridization results of human

probes onto mule chromosomes are in agreement with those of the horse probes on chromosomes of human and donkey.

Discussion

We have made a full set of chromosome-specific paint probes for the horse and these probes have been assigned to chromosomes of the horse, donkey, mule and human by fluorescence *in-situ* hybridization. Based on the hybridization results, we have established genome-wide comparative chromosome maps between horse and human, human and donkey, and horse and donkey. These maps should benefit the ongoing equine genome project and positional candidate gene cloning studies. The rapid progress in the equine genome project has made the horse genome the

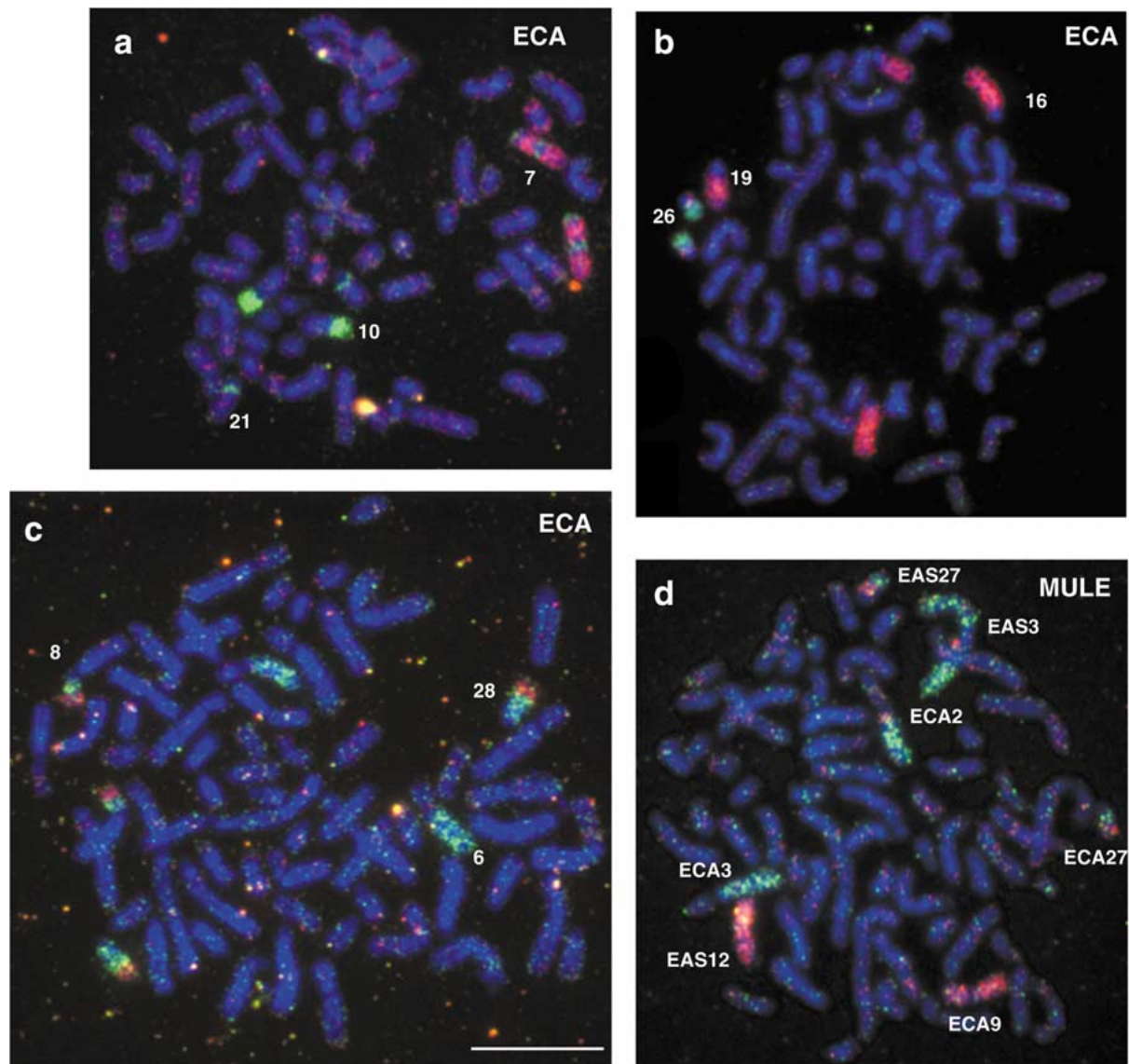


Figure 5. Examples of reciprocal painting between human and equine. (a) HSA 11 (red) and HSA 19 (green), (b) HSA 3 (red) and HSA 21 (green), and (c) HSA 12 (green) and HSA 22 (red) hybridized to horse metaphases (ECA); (d) HSA 4 (green) and HSA 8 (red) probes painted onto mule metaphases. Note that only one of the two homologues that are painted is numbered. Scale bar = 10 μ l.

appropriate reference for comparative cytogenetic and genomic studies of perissodactyls and such studies will benefit greatly from the availability of the full set of horse paints.

Human–horse/donkey map

A series of comparative maps between human and horse have been established by various

approaches including comparative painting with human paints, and comparative gene and RH mapping (Raudsepp *et al.* 1996, Milenkovic 2002, Chowdhary *et al.* 2003). But the human–donkey map reported here represents the first genome-wide comparative genome map between human and donkey based on painting homologies. The human–horse reciprocal painting defines the distribution, patterns and boundaries of

homologous segments conserved between human and horse and has improved the resolution of the previous map based on incomplete unidirectional painting (i.e. human to horse). In doing so, it shifts resolution from the whole-human chromosome to the subchromosomal level.

Our horse-human comparative chromosome map is largely in agreement with that suggested by comparative mapping of Type I markers (Chowdhary *et al.* 2003). In addition to verifying previously established associations, our data identify some discrepancies in the correspondence between human and horse chromosomes (Table 1). For instance, previous studies involving painting with human probes have shown that horse chromosome 1 (from 1pter to 1qter) is homologous to HSA 22-10-2-15-12-15-14 (Raudsepp *et al.* 1996) but recent RH mapping suggests that ECA1 = HSA22-10-15-14 (Chowdhary *et al.* 2003). In contrast, our reciprocal painting clearly demonstrates that ECA1 = HSA10-1-10-14-15 and we failed to detect any homologies between HSA22 and ECA 1. In addition, some subtle homologous segments defined by single-gene homologies, such as HSA22 on ECA 1 and ECA 5, and HSA9 on ECA 19 as shown on the latest RH map (Chowdhary *et al.* 2003), were not detected by reciprocal painting.

Our results demonstrate that the horse has retained the conserved ancestral syntenies HSA

3/21 and HSA 4/8 on ECA 26 and ECA 27, respectively (Figure 5b, d). Previously, painting with human probes and comparative gene and RH mapping all failed to detect the HSA 3/21 and HSA 4/8 syntenic relationships in the horse genome. This finding has important implications for our understanding of mammalian genome evolution. It shows that the ancestral syntenies HSA 3/21 and HSA 4/8, although very short in length and thus readily missed, have been maintained in both the domestic horse and Hartmann's mountain zebra (Richard *et al.* 2001). In addition, the homology we demonstrated between the proximal part of ECA 21 and HSA 19 (Figure 5a) represents another segment undetected in previous studies. It is noteworthy the HSA 5/19 syntenic association found on ECA 21 has also been reported in cattle, pig, dolphin and Indian muntjac. The HSA5/19 association therefore appears to represent a shared, derived synteny association specific to cetartiodactyls and perissodactyls (the Euungulata or true ungulates *sensu* Waddell 2001) as reported previously (Yang *et al.* 2003b). Moreover, by referencing our comparisons to the human, our data enable the horse and donkey genomes to be linked to the comparative maps that exist between human and the representative species of 12 orders of eutherian mammals which have already been established (see Chowdhary & Raudsepp 2001 for review, Yang *et al.* 2003a), thereby contributing to a broader understanding of genome evolution of mammals.

Horse-donkey comparative chromosome map

The use of the mule metaphases, within which the horse and donkey homologues are at the same level of contraction, assisted greatly in sharpening the accuracy of the comparative map. Our data represent a significant improvement on results obtained from comparative painting with probes from only the 15 microdissected horse chromosomes (ECA1-13, X and Y; Raudsepp & Chowdhary 1999) by extending the coverage of the homology map to entire horse and donkey genomes. Although comparative chromosome painting is limited in its ability to detect inversions, combined painting and banding pattern comparisons allow a genome-wide scrutiny of the inter- and intrachromosomal

Table 1. Summary of earlier chromosomal assignments between human and horse and refinements suggested by the present study.

Horse chromosomes	Chromosome painting (HSA-ECA) (Raudsepp <i>et al.</i> 1996)	RH map (Chowdhary <i>et al.</i> 2003)	Reciprocal painting (this study)
1	22/10-cen-2/15/12/15/14	22/10-Cen-15/14	10-cen-1/10/15/14
2	1-cen-4	2/1-cen-4/8/4/1/4	1-cen-8/4
6p	?	12	12
12	?	11	11
13	?	7/16-cen7	16-cen-16/7
21	5	5	Cen-19/5
26	21	21	Cen-3/21
27	?	4	Cen-4-8
31	?	6	6qter

? = no hybridization to human.

rearrangements that differentiate the karyotypes of the horse and donkey. This greatly assists in identifying the probably meiotic causes that are responsible for the sterility of horse and donkey interspecific hybrids.

As demonstrated in Figure 3, most of the homologous euchromatic segments between horse and donkey show highly conserved banding patterns; the regions which lack homologies correspond to centromeric and telomeric regions that consist mainly of heterochromatin. In the donkey genome, the synteny of six horse chromosomes (ECA 2, 3, 5, 6, 8 and 10) have each broken into two segments that form separate donkey chromosomes or part of a donkey chromosome. In contrast, relatively few inversions characterize the horse chromosomes, with ECA 4 and 31 being the most obvious examples of this type of intrachromosomal rearrangement. The painting patterns of ECA 4 and ECA 31 on EAS 1 suggest that the donkey chromosome may have evolved via one chromosomal fusion of horse 4 and 31 followed by one pericentric inversion and the subsequent amplification of centromeric heterochromatin. In addition, ECA7 and its donkey homologue (EAS20) differ by one pericentric inversion, which is clearly evident from the painting patterns of the HSA 11 and 19 probes. Furthermore, centromere repositions or subtle inversions could also have

been involved during the karyotypic divergence of horse and donkey. For instance, the acrocentric horse chromosomes ECA 14, 15, 17 and 22 correspond to the biarmed donkey chromosomes EAS 9, 6, 11 and 15 respectively. In spite of the difference in centromere positions between the horse and donkey homologues, these interspecific homologues have largely conserved G-banding patterns, an indication of the possible involvement of centromere repositions or alternatively subtle inversions that are beyond the resolution provided by FISH. Our findings contrast with the previous report that only about 40% of the asine–equine homologues defined by painting showed correspondence in banding (Raudsepp & Chowdhary 1999). This discrepancy may have been caused by errors in chromosomal identification and signal assignment. The Raudsepp & Chowdhary study also failed to detect the disruption of ECA 4 synteny by ECA 31 on donkey EAS 1, and ECA 8q was erroneously assigned to EAS 5p (instead of ECA 7q15-pter as shown in the present study). The genome-wide chromosomal correspondence depicted in Figure 3 is based on sequential G-banding and multicolour FISH, and has been further verified by painting with human probes. Ignoring heterochromatic variation, it would require 6 fissions, 10 fusions, at least 2 inversions and several centromere repositions to convert the

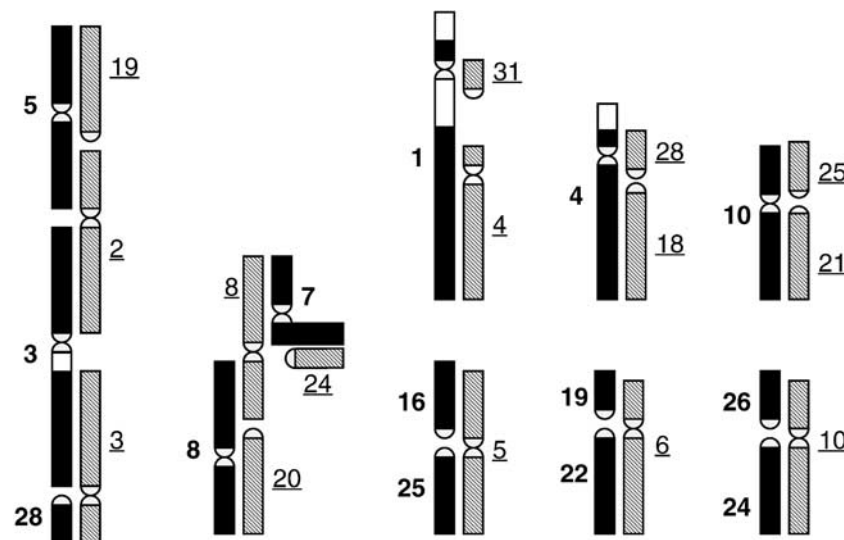


Figure 6. Multivalents anticipated to be formed during the gametogenesis of fertile mules. Donkey chromosomes are solid, horse chromosomes are cross-hatched, and heterochromatic regions are open.

horse karyotype into that of the donkey. Furthermore, the failure of paints from the acrocentric horse chromosomes to delineate the centromeres of their corresponding donkey acrocentrics indicates rapid divergence of satellite DNA sequence among the horse and donkey acrocentric homologues.

Our results provide further insight into the sterility and fertility of horse–donkey interspecific hybrids. Mules and hinnies, the hybrid offspring between the horse and donkey, have been a source of fascination for cytogeneticists, particularly in light of the well-documented reports of fertile mules giving birth to viable offspring (Ryder *et al.* 1985, Rong *et al.* 1988, Zong & Fan 1989). Complex structural chromosomal rearrangements that occurred during speciation are long believed to have caused meiotic breakdown in hybrid offspring leading to their sterility. Banding comparisons alone have so far failed to provide adequate resolution for deciphering these rearrangements. Despite the complex rearrangement distinguishing the two species, normal meiotic division during gametogenesis has occasionally been observed, suggesting that meiotic pairing is possible (Taylor & Short 1973, Chandley *et al.* 1974). The establishment of genome-wide homologues enables us to deduce the possible configuration of meiotic pairing in fertile mules (Figure 6). One hexavalent (involving ECA 2, 3, 19, and EAS 3, 5, and 28), one pentavalent (involving ECA 8, 20, 28, and EAS 7 and 8), six trivalents, and 17 bivalents would result in the meiotic prophase of fertile mules if homologous pairing does occur. One can further infer that the chromosome numbers of the genetically balanced eggs or sperm should range from $n = 28$ to $n = 35$, assuming random segregation. The diploid number of offspring of fertile mules (when backcrossed to either donkey or horse) could, therefore, vary from $2n = 59$ to $2n = 67$, with the $2n$ of the majority falling into the range of 62–64. This prediction is in agreement with the cases so far reported (Ryder *et al.* 1985, Rong *et al.* 1988, Zong & Fan 1989) although a mule offspring with $2n = 60$ has been reported in China (Zong & Fan 1989). In conclusion, the availability of horse-chromosome-specific painting probes provides the discrimination necessary for the unequivocal characterization of the chromosomal

complements of the offspring of fertile mules, an aspect of equine reproduction that will be the focus of ongoing research in our laboratory.

Acknowledgements

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